EXHIBIT B

2021

ENHANCED REPLICATION OF ATTENUATED HSV-1 IN IRRADIATED HUMAN GLIOMA XENOGRAFTS

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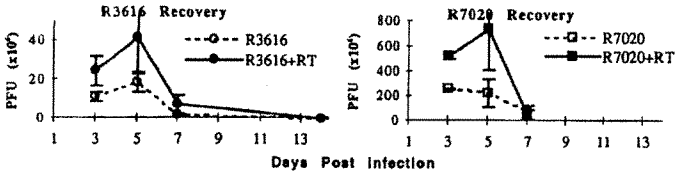
Purpose: Previously we had shown that combining ionizing radiation (IR) with attenuated replication competent HSV-1 (R3616) significantly increased glioma xenograft eradication compared to IR or virus alone. One hypothesis is that IR induces cell factors that contribute to augment viral replication thereby increasing the efficacy of attenuated HSV-1. The purpose of this study was to examine if IR altered viral replication of attenuated HSV-1 in glioma xenografts

Material and Methods: Human U-87MG glioma cells were grown in the hindlimb of athymic mice and grown to >200 mm^3. Tumors were infected with $2x10^7$ plaque forming units (pfu) of R3616 ($\gamma_1$34.5$^-$) or R7020 (multimutated, $\gamma_1$34.5$^+$) on day 0 and irradiated with 20 Gy on day 1 and 25 Gy on day 2. Tumors were harvested 3, 5, 7, and 14 days after viral injection. Tumors were homogenized and sonnicated. Serial dilutions of tumor extract were overlaid on Vero cells to determine the number of pfu. In addition, in-situ hybridization to HSV-1 DNA was performed on tumors harvested at day 7.

Results: In-situ hybridization revealed larger numbers of glial cells infected with HSV along with a greater distribution in the irradiated tumors compared to non-irradiated tumors. We next quantified viral particles in infected tumors +/- IR:



Conclusion: Herein we demonstrate radiation enhanced viral replication as one of the interactive effects of combining IR and attenuated HSV in treating glioma xenografts and a potential therapeutic motif in the treatment of gliomas. To reduce normal tissue toxicity of HSV in glioma therapy, viruses must be attenuated. However, attenuating the virus compromises its replication and thus its potential efficacy. Our results indicate that IR augments the amount of virus recovered from human glioma xenografts for up to 3 days post IR. The results do not appear to be related to a specific mutation in the herpes genome but rather to herpes viruses in general. Yields of R7020 were greater than R3616 since R7020 is less attenuated than R3616. IR provides a conducive local environment for viral replication and can specifically target enhanced viral replication to the irradiated tumor bed. We hypothesize ionizing radiation induces cellular gene(s) involved in glioma DNA repair that interact in the viral replication cycle to enhance viral replication. This effect is transient in that as the levels of the cellular induced gene(s) fall viral replication is no longer enhanced, and attenuated viruses diminish the threat of systemic toxicities.

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MOLECULAR REQUIREMENTS FOR RADIATION-ACTIVATED RECOMBINATION

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Purpose/Objective: The major stumbling block to successful gene therapy today is poor gene transfer. We hypothesized that ionizing radiation might activate cellular recombination, and so improve stable gene transfer. We further hypothesized that known DNA-damage-repair proteins might also be important in radiation-activated recombination.

Materials and Methods: The effect of irradiation on stable gene transfer efficiency was determined in human (A549 and 39F) and rodent (NIH/3T3) cell lines. Continuous low dose rate and multiple radiation fractions were also tested. Nuclear extracts were made and the effect of irradiation on inter-plasmid recombination/ligation determined. Multiple DNA damage-repair deficient cell lines were tested for radiation-activated recombination.

Results: A significant radiation dose-dependent improvement in stable plasmid transfection (by as much as 1300 fold) is demonstrated in neoplastic and primary cells. An improvement in transient plasmid transfection is also seen, with as much as 85% of cells transiently expressing b-galactosidase (20-50 fold improvement). Stable transfection is only improved for linearized or nicked plasmids. Cells have improved gene transfer for at least 96 hours after irradiation. Both fractionated and continuous low dose rate irradiation are effective at improving stable gene transfer in mammalian cells, thus making relatively high radiation dose delivery clinically feasible. Inter-plasmid recombination is radiation dose dependent in nuclear extract assays, and the type of overhang (3', 5' or blunt end) significantly affects recombination efficiency and the type of product. The most common end-joining activity involves filling-in of the overhang followed by blunt end ligation.

Adenovirus is a linear, double stranded DNA virus. We demonstrate that adenoviral infection efficiency is increased by irradiation. The duration of transgene expression is lengthened because the virus integrates with high efficiency (~10% of treated cells) into cellular DNA.

The mechanism of radiation enhanced stable gene transfer requires effector proteins to accomplish the recombination. The Ku proteins, which are required for V(D)J recombination, account for at least 90% of radiation induced recombination. There is also an absolute requirement for the Ataxia Telangiectasia gene (ATM) for any radiation induced recombination to occur, although the transfection efficiency in unirradiated cells is unaffected by ATM. Removal of p53 by transfection of E6 (Human Papilloma Virus) significantly inhibits radiation activated recombination, and this is confirmed in nuclear extract recombination assays.

Conclusions: Ionizing radiation activates a recombination pathway which may be useful in gene therapy. The molecular mechanism of radiation activated recombination requires a number of DNA-damage-repair proteins.